Inactivation by Nitrogen Mustard of Single- and Double-Stranded DNA and RNA Bacteriophages

Abstract. *Nitrogen mustard inactivates bacteriophage containing single-stranded DNA and RNA as well as double-stranded DNA. Inactivation may occur by intrastrand cross-linkage in DNA or RNA as well as by interstrand cross-linkage between complementary strands of DNA.*

The studies of Brookes and Lawley (*1, 2*) and others (*3*) have led to the conclusion that the biological effect of bifunctional alkylating agents such as nitrogen mustard is due to the formation of intermolecular cross-linkages between the strands of double-stranded DNA (interstrand cross-linkage). The reactive site appears to be at guanine, since diguaninyl-7 derivatives are isolated from reactions with DNA or guanine nucleotides (*1, 2, 4*). Loveless (*5*) reported that bacteriophage T2, a double-stranded DNA phage, is vastly more sensitive to inactivation by bifunctional alkylating agents than the single-stranded DNA phage ϕX174 is. These findings imply that reaction occurs primarily with double-stranded DNA molecules.

To elucidate further the relation of strandedness to susceptibility to alkylating agents, we examined the effects of a series of such compounds; five types of bacteriophage, single as well as double-stranded DNA and RNA, were used. Our results contribute to existing information on the mechanism of action of alkylating agents on bacteriophages in their role as models of DNA and RNA molecules.

Bacteriophage T2, a tadpole-shaped double-stranded DNA phage, was assayed on *Escherichia coli* B; bacteriophage P22, a smaller, short-tailed double-stranded DNA phage was assayed on *Salmonella typhimurium* Q1 (*6*); bacteriophage S13, a spherical, single-stranded DNA phage was assayed on *S. typhimurium* St/22; bacteriophage f1, a rod-shaped, single-stranded DNA phage (*7*) was assayed on the HfrC strain of *E. coli* K12; and MS2, a single-stranded RNA phage, was assayed on the same strain.

The alkylating agents were 2,2′-dichloro-*N*-methyldiethylamine (nitrogen mustard, NSC-762), a bifunctional agent, and two monofunctional agents, 2-chloroethylamine (NSC-10871) and 2-chloro-*N*,*N*-dimethylethylamine (NSC-1917).

Bacteriophage was suspended at a concentration of 10^7 particles per milliliter in buffered saline (0.07*M* phosphate in 0.1*M* NaCl at *p*H 7.0, containing 1 m*M* magnesium sulfate). One part of a solution of the alkylating agent in buffered saline was added to nine parts of the suspension of phage. All manipulations were carried out at room temperature. Periodically, 0.1-ml portions of the mixture were withdrawn, diluted to 10 ml with buffered saline to stop the action of residual agent; this mixture was further diluted 10- and 100-fold. Portions (0.25 ml) of each diluted sample were added to 0.1 ml of host bacteria that had been cultured in nutrient broth. Two milliliters of soft agar (0.8 percent Difco Bacto-agar in Difco nutrient broth) was added to the phage–bacteria mixture and poured over hard nutrient agar (1.5 percent agar) plates. After incubation at 37°C for 18 hours, inactivation of the phage was determined by loss of plaque-forming activity.

In order to maintain the temperature, concentration of chemicals, and *p*H at identical conditions for comparisons of effect on different phages, a method was designed in which two or three phages, each requiring different indicator hosts, were mixed in the same solutions. For example, nitrogen mustard was added to three tubes, each containing, respectively, phages P22, S13, and a mixture of P22 and S13. The curves for the subsequent inactivation of each phage were identical, an indication that mixing of phages did not interfere with the validity of the assays.

The two monofunctional agents (2-chloroethylamine and 2-chloro-*N*,*N*-dimethylethylamine), in concentrations ranging from 2.5 m*M* to 126 m*M*, did not inhibit phage activity during the 60 minutes of exposure. In contrast the bifunctional nitrogen mustards (2,2′-dichloro-*N*-ethyldiethylamine) markedly inactivated all five phage types, and with all concentrations of nitrogen mustard ranging from 0.6 m*M* to 13 m*M*; at 6.4 m*M* (1 mg/ml) of nitrogen mustard the velocity constants of inactivation were: in P22, 0.43 ± 0.04; T2, 0.54 ± 0.05; S13, 0.29 ± 0.02; f1, 0.31 ± 0.03; MS2, 0.35 ± 0.01 min^{-1}. Inactivation was most rapid with the two double-stranded DNA phages, T2 and P22 (Fig. 1). The linear inactivation curve showed no evident lag period for T2, and only a short lag with P22. Similar inactivation was also observed with single-stranded phages, but the rates were slower. These inactivation curves are reproducible for all phages tested. Since we tested two or three types of phages in admixture, comparisons between the inactivation curves of these phages are reliable. The respective velocity constants indicate that the double-stranded phages are inactivated about 1.5 times more quickly than the single-stranded phages are. The rates of inactivation of the single-stranded phages were approximately the same, but the spherical phages, S13 and MS2, always have short initial lag periods of approximately 1 to 2 minutes.

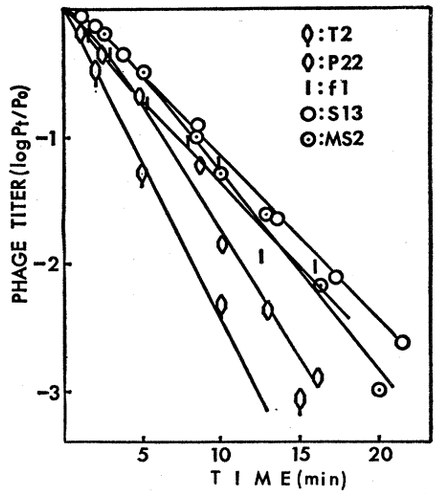

Fig. 1. Inactivation of bacteriophages by nitrogen mustard (6.4 m*M*). In order to maintain the temperature, concentration of chemicals, and *p*H at identical conditions for comparisons of effect on different phages, two or three phages were mixed in buffered saline, *p*H 7. Inactivation was determined by assaying at intervals each phage.

Inactivation of phage by bifunctional, but not by monofunctional, alkylating agents supports the conclusion of Brookes and Lawley (*1*) that inactivation requires combination at two reactive sites. Since an RNA phage, MS2, was inactivated at approximately the same rate as the DNA phages f1 and S13, inactivation by an alkylating agent is not dependent

on the nature of the sugar moiety, nor on the presence of either thymine or uracil among the nucleic acid bases.

Inactivation of single-stranded DNA phage, S13, as well as of double-stranded DNA phages T2 and P22, suggests that alkylating agents can link not only across complementary strands (interstrand cross-linkage), but also across reactive sites on a single strand (intrastrand cross-linkage). The relatively faster rate of inactivation of the double-stranded, as compared with single-stranded phages, may indicate that both types of cross-linkage are possible in the double-stranded DNA phage. The spatial arrangements of DNA in the double-stranded T2 and P22, as well as that in the single-stranded spherical S13, are unknown. It is conceivable that intrastrand cross linking might occur across sections brought close together by folding. However, this would not be true for rod-shaped f1 phage, which seemingly exists as a single, straight helix, similar to the RNA of tobacco mosaic virus.

With a double-stranded DNA phage P22, a single-stranded DNA phage S13, and RNA phage MS2, there are short lag periods occurring during the initial 1 to 2 minutes of the reaction. This may represent either the time necessary to complete a two-stage reaction or a delay in the alkylating agent's penetrating the protein coat of the phage particle. Commings and Kozloff (*8*) reported that raising *p*H increases the permeability of phage protein coat. They showed that at high *p*H large molecules such as methylene blue can penetrate the protein coat and gain access to DNA. In our experiment at high *p*H the inactivation rate by nitrogen mustard was accelerated for all phages. Moreover, raising *p*H decreases the initial lag period (*9*). This observation suggests that the initial lag period of inactivation is dependent on the permeability of the protein coat.

That other mechanisms, such as an alkylation of amino acids in the protein coat, participate in phage inactivation, cannot be ruled out. Two amino acids, histidine and lysine, are highly reactive with alkylating agents (*10*). However, at *p*H 7.0, under the conditions of these investigations, lysine is not reactive (*10*), and the protein coat of phage MS2 does not contain histidine (*11*). From the above information the reaction between amino acids of protein coat and nitrogen mustard does not seem to play an important role in the inactivation of phage.

NOBUTO YAMAMOTO
TATSURO NAITO*
Fels Research Institute and
Department of Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania

References and Notes

1. P. Brookes and P. D. Lawley, *Biochem. J.* **80**, 496 (1961); *ibid.* **89**, 138 (1963).
2. ———, *J. Chem. Soc.* **1961**, 3923 (1961).
3. P. Geiduscheck, *Proc. Natl. Acad. Sci. U.S.* **47**, 950 (1961); B. Papirmeister, U.S. Army CRDL Special Publications, 2-45 (1961); A. Loveless and J. C. Stock, *Proc. Roy. Soc. London Ser. B* **150**, 423 (1961).
4. P. Brookes, in *Chemotherapy of Cancer*, P. A. Plattner, Ed. (Elsevier, New York, 1964).
5. A. Loveless, *Ann. Rept. Brit. Empire Cancer Campaign* **40**, 19 (1962).
6. J. S. K. Boyd, *Nature* **4525**, 141 (1956).
7. N. D. Zinder, R. C. Valentine, M. Roger, W. Stoeckenius, *Virology* **20**, 638 (1963).
8. D. J. Commings and L. M. Kozloff, *J. Mol. Biol.* **5**, 50 (1962).
9. N. Yamamoto, T. Naito, M. B. Shimkin, in preparation.
10. R. L. Heinrikson, W. H. Stein, S. Moore, *Federation Proc.* **24**, 592 (1965).
11. Y. Ohtaka and S. Spiegelman, *Science* **142**, 493 (1963).
12. Supported in part by grants from NIH (Ca07174-02) and NSF (GB-2680). We thank Cancer Chemotherapy National Service Center, National Cancer Institute, for a supply of the NSC chemicals; and Dr. S. Weinhouse and Dr. M. B. Shimkin for suggestions throughout this study.
* On leave from Department of Bacteriology, Nagasaki University School of Medicine, Nagasaki, Japan.

29 September 1965

Susceptibility of the Gibbon Hylobates lar to Falciparum Malaria

Abstract. *The splenectomized juvenile gibbon,* Hylobates lar, *is susceptible to infection with trophozoites of* Plasmodium falciparum. *All subjects in a group of six animals that were injected with freshly collected blood from four infected humans in Saraburi Province, Thailand, developed parasitemia 2 to 20 days later; infections were transmitted from one of these subjects to three clean gibbons.*

The white-handed gibbon (*Hylobates lar*) is a potential laboratory tool for the study of *Plasmodium falciparum* infections. Although the splenectomized chimpanzee (*Pan satyrus*) can be readily infected with falciparum malaria (*1, 2*), rapid decimation of chimpanzee populations, problems of animal management, and common parasitism with three species of malarial parasites indicate that another animal might better serve as an experimental host for this parasite.

Young gibbons (weighing 1.75 to 2.75 kg) were splenectomized by SEATO Medical Research Laboratory personnel between 16 April and 10 June. For radical cure of possible latent malarial infections, these animals were placed on a chloroquine and primaquine regimen (*3*). During the period of experimental work the gibbons were housed in mosquito-proof quarters.

Six splenectomized gibbons (P-1, P-3, P-7, P-8, P-11, and P-13) were inoculated with samples of blood from patients at the Prabuddahbet Malaria Clinic with *Plasmodium falciparum* in-

Table 1. Infection of gibbons, *Hylobates lar,* with asexual *Plasmodium falciparum.* All animals except P-12 were splenectomized prior to inoculation.

Gibbon No.	Prepatent period (days)	Day of peak parasitemia	Days of continual parasitemia	Highest No. parasites per mm³ of blood
P-1	5	35	22 to 39 +	143,500
P-2	5	12	5 to 22 +	241,800
P-3	17	25	25 to 26	11,750
P-7	20	35	23 to 46 +	38,000
P-8	2	37	28 to 39 +	15,600
P-11	4	18	18 to 19	310
P-12	5	14	5 to 6	1,400
P-13	2	17	2 to 54 +	57,000
S-2	4	12	4 to 22 +	45,200